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Induction of Autophagy Restores the Loss of Sevoflurane Cardiac Preconditioning Seen with Prolonged Ischemic Insult

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Short running title: Sevoflurane preconditioning and autophagy

Abstract

Background: Sevoflurane preconditioning (SPC) against myocardial ischemia-reperfusion injury is lost if the ischemic insult is too long. Emerging evidence suggests that induction of autophagy may also confer cardioprotection against ischemia-reperfusion injury. We examined whether induction of autophagy prolongs SPC protection during a longer ischemic insult.

Methods: Isolated guinea pigs hearts were subjected to 30 or 45 min ischemia, followed by 120 min reperfusion (control). Anesthetic preconditioning was elicited with 2% sevoflurane for ten minutes prior to ischemia (SEVO-30, SEVO-45). Chloramphenicol (autophagy upregulator, 300 μM) was administered starting 20 min before ischemia and throughout reperfusion in SEVO-45 (SEVO-45+CAP). To inhibit autophagy, 3-methyladenine (3MA:10 μM) was administered during sevoflurane administration in SEVO-45+CAP. Infarct size was determined by triphenyltetrazolium chloride stain. Tissue samples were obtained before ischemia to determine autophagy-related protein (microtubule-associated protein light chain I and II: LC3-I, II), Akt and glycogen synthase kinase 3β (GSK3β) expression using Western blot analysis. The effect of autophagy on calcium-induced mitochondrial permeability transition pore (MPTP) opening in isolated calcein-loaded mitochondria was assessed. Electron microscopy was used to detect autophagosomes.

Results: Infarct size was significantly reduced in SEVO-30, but not in SEVO-45. Chloramphenicol restored SPC lost by 45 min ischemia. There were more abundant autophagozomes and LC3-II expression was significantly increased in SEVO-45+CAP. Induction of autophagy before ischemia enhanced GSK3β phosphorylation and inhibition of calcium-induced MPTP opening. These effects were abolished by 3MA.

Conclusions: Pre-ischemic induction of autophagy restores SPC lost by longer ischemic insult. This effect is associated with enhanced inhibition of MPTP by autophagy.

Key Words:
autophagy, chloramphenicol, ischemia-reperfusion, preconditioning, sevoflurane

Introduction

Experimental[1-3] and clinical studies[4,5] have demonstrated the use of volatile anesthetics as an additional therapeutic approach in the care of patients at risk of developing perioperative cardiac complications; known as volatile anesthetic preconditioning.[6,7] The washout time of volatile anesthetics prior to ischemia[8] and the duration of sustained ischemia[9,10] have been shown to be crucial to successful volatile anesthetic cardioprotection. Kevin et al. demonstrated that sevoflurane cardioprotection is restricted to a range of ischemic durations of 25-40 min in isolated guinea pig hearts.[9] Similarly, the neuroprotective effects of clinically relevant sevoflurane concentrations was observed only for ischemia of ≤30 minutes.[10] Finding strategies that prolong cardioprotection by sevoflurane preconditioning during longer ischemic insults could be clinically useful.

Autophagy is a catabolic process through which damaged or long-lived protein and organelles are degraded using lysosomal degradative pathways. This is an evolutionally conserved process crucial for normal tissue homeostasis. Autophagy is considered one mode of cell death, termed “autophagic cell death”. Certainly, necrosis and apoptosis contribute to cardiomyocyte death in ischemia-reperfusion injury. However, accumulating evidence suggests that autophagy is stimulated by ischemia and may contribute to cardiomyocyte survival.[12,13] Yet, excessive self-digestion and degradation of essential cellular components by autophagy could be detrimental.[14] To date, it is unclear whether upregulation of autophagy is protective or detrimental for ischemic myocardium.

Autophagy is triggered by opening of mitochondrial permeability transition pores (MPTP), which also play an important role in myocardial ischemia-reperfusion injury.[15] Studies have shown that inactivation of glycogen synthase kinase 3β (GSK3β) by

phosphorylation at Ser[9] inhibits MPTP opening and protects cardiomyocytes against ischemia-reperfusion injury.[16] It is not known whether pre-ischemic induction of autophagy affects MPTP. We hypothesized that pre-ischemic induction of autophagy restores loss of sevoflurane preconditioning seen with prolonged ischemic insult and this effect is associated with MPTP opening. Elucidating the role of autophagy in ischemia-reperfusion injury and finding strategies that prolong cardioprotection by sevoflurane preconditioning during longer ischemic insults could prove clinically useful in patients at cardiovascular risk during perioperative periods.

Methods

This study was conducted in accordance with the Guidelines for Animal Research at Osaka Dental University with approval of the Animal Experiment Committee of Osaka Dental University, Osaka, Japan. These guidelines conform to the Guide for the Care and Use of Laboratory Animals from the National Academy of Science. Male Hartley guinea pigs were fed Lab Diet (RC4, Oriental Yeast, Tokyo, Japan) and water *ad libitum.* Chloramphenicol was purchased from Calbiochem (La Jolla, CA, USA) to induce autophagy. 3-methyladenine (3MA) was purchased from Sigma Aldrich (Ann Arbor, MI, USA) to inhibit autophagy.

Isolated Heart Perfusion and Measurement of Function

Guinea pigs (550-700g; 12-13 weeks old) received heparin (1000 units intraperitoneally), then were anesthetized with pentobarbital (60 mg^{-1}·kg^{-1}, intraperitoneally). Hearts were excised and arrested in cold iso-osmotic saline containing 20 mM KCl. The aorta was cannulated and hearts were perfused at 70 mmHg on a nonrecirculating isovolumic perfusion apparatus, using Krebs-Henseleit perfusate, and paced at 240 beats/min.[3] Left ventricular developed pressure (LVDP) was measured using a 2.5 French, high-fidelity micromanometer (Nihon-Kohden, Tokyo, Japan) passed into a compliant latex balloon, inserted into the left ventricle, and recorded on a PowerLab 2/20 Data Recording System (ADInstruments, Hayward, Australia). The balloon was connected to a Y-adapter with one end used to advance the micromanometer and the other to fill the left ventricular balloon with bubble-free water to an end-diastolic pressure (LVEDP) of 10 mmHg. Coronary flow (CF) (ml·min^{-1}·g^{-1}) was measured by collecting effluent. Global ischemia

was achieved by clamping the aortic inflow line. During ischemia, hearts were maintained at 37 °C by enclosure in a water-jacketed air chamber. Warmed perfusate in the lower part of the chamber saturated the air with humidity and prevented cooling by evaporation. Heart temperature was continuously monitored by a digital thermometer (PTW-100A, Unique Medical, Tokyo, Japan). Sevoflurane was insufflated passing the 95%O_2/5%CO_2 gas mixture through a calibrated vaporizer (ACOMA, Tokyo, Japan). Coronary perfusate samples were collected anaerobically from the aortic cannula for measurement of sevoflurane concentration by an organic vapor sensor (OSP, Saitama, Japan).

Experimental Protocol

Animals were assigned 10 groups (n=8 each; Figure 1). After 20 min equilibration, baseline LVDP, LVEDP and CF were recorded. Hearts were subjected to 30 min (control; CTL-30) or 45 min (control; CTL-45) ischemia followed by 120 min reperfusion. Anesthetic preconditioning was elicited by administration of sevoflurane (2%) for 10 min followed by 10 min washout before 30 min or 45 min ischemia (SEVO-30, SEVO-45). For induction of autophagy, chloramphenicol (300 μM) was administered starting 20 min before 45 min ischemia and throughout reperfusion in sevoflurane-treated (SEVO-45+CAP) or non-sevoflurane-treated animals (CAP-45) hearts. To inhibit autophagy, 3MA (10 μM) was administered during sevoflurane administration for 20 min in SEVO-45+CAP (SEVO-45+CAP+3MA) and its vehicle (saline) in CTL-45 (CTL-45+3MA) (Figure 1). Chloramphenicol was dissolved in distilled water and 3MA was dissolved in 0.04% ethanol, to final concentrations of 300 μM and 10 μM, respectively.

Determination of Myocardial Infarct Size

At experiment end, hearts were frozen at –80 °C for 15 min, then sliced into 2 mm thick transverse sections from apex to base (6 slices/heart). After defrosting, slices were incubated at 37 °C with 1% triphenyltetrazolium chloride (Sigma Chemicals, USA) photographed, and necrotic area was determined using Adobe Photoshop® CS (Adobe, San Jose, CA, USA) as previously described.[3]

Western Blot Analysis

Separate experiments were performed (n=4 per group) to examine expression of autophagy-related protein (microtubule-associated protein light chain I and II: LC3-I, II), Akt, GSK3β and cyclophilin D. Myocardial tissue samples were collected before ischemia, and homogenized in ice-cold homogenizing buffer as previously described.[3] Homogenate was centrifuged at 1000g for 5 min at 4 °C. Supernatant was re-centrifuged at 10000g for 15 min at 4 °C to obtain cytosolic fraction. The pellet was designated as crude mitochondrial fraction and was resuspended in homogenizing buffer with 1% TritonX-100, incubated on ice for 1hr, then re-centrifuged at 10000g for 15 min at 4 °C. The resulting supernatant was used as mitochondrial fraction. Protein concentration was estimated with a Bradford assay. Equivalent amounts (20 μg) of protein samples were loaded and separated on 10% SDS-PAGE gradient gel, then electrically transferred overnight to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), membranes were incubated for 2 h at 4 °C in TBS-T containing 5% skim milk and overnight with 1:200-1000 dilution of rabbit primary antibody for LC3 (Medical & Biological Laboratories Co LTD, MA, USA), phospho Akt (Ser47), phospho GSK3β (Ser9)

(Cell Signaling TECHNOLOGY, Boston, MA, USA) and cyclophilin D (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were incubated with a 1:1000 dilution of horseradish peroxidase–labeled anti-rabbit immunoglobulin G (NA 934V, GE Healthcare, Buckinghamshire, United Kingdom). The same blot was stripped and re-blotted with α-tubulin antibodies (Santa Cruz Biotechnology), total Akt and GSK3β (Cell Signaling TECHNOLOGY). Bound antibody signals were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) and visualized using VersaDoc 5000 Imaging System (Bio-Rad, Hercules, CA). Quantitative analysis of band densities was performed with Quantity One software (Bio-Rad). Results are presented as the ratio of phospho Akt and phospho GSK3β to total (including non-phosphorylated and phosphorylated protein) Akt and GSK3β, respectively.

Studies in isolated cardiac mitochondria

To investigate involvement of MPTP in cardioprotection by autophagy, mitochondria were isolated (n=4 per group). After perfused and treated with sevoflurane, chloramphenicol and 3MA, isolated hearts were removed from the Langendorff apparatus, and homogenized in ice-cold MSTEB buffer as previously described.[17] Extracted mitochondria were diluted in ice-cold respiratory buffer and incubated with 1μM calcein-AM (Invitrogen Molecular Probes, Carlsbad, USA) for 15 min at room temperature. Calcein-AM readily enters the mitochondria and is trapped in the matrix in its free form, which is fluorescent. After calcein was trapped in mitochondria, mitochondria were washed by KCl buffer.[17] Calcein-loaded mitochondria were treated with 50 and 150 μM Ca^{++} per milligram of protein, and were incubated for 10 min at room temperature. Mitochondrial fluorescences were acquired. Flow

cytometric analysis was performed on FACS calibur™ (Becton Dickinson, Franklin lakes, NJ). Mitochondria labeled with calcein-AM were analyzed by flow cytometry in an instrument equipped with a 488 nm excitation source.

Electron Microscopy

To confirm induction of autophagy, cardiac specimens were obtained before sustained ischemia. They were fixed with phosphate-buffered 2.5 % glutaraldehyde (pH 7.4) and were postfixed with 1 % osmium tetroxide, after which they were conventionally prepared for transmission electron microscopy (H-800, Hitachi, Tokyo, Japan).

Statistical Analysis

Data are expressed as mean±SD. Statistical power analysis revealed a sample size of n=8 would provide sufficient power (0.8) to detect a difference between mean infarct size indices of 15 % (SD=9, α=0.05). A group size of n=4 was used for Western blot and calcein studies to provide a power of 0.8 to detect a difference between means of 20% (SD=10, α=0.05). Hemodynamic data were tested for normal distribution and subsequently analyzed by a two-factor repeated-measures analysis of variance for time and treatment. If an overall difference between variables was observed, comparisons were performed as one-way ANOVA followed by Tukey's post-hoc test for inter-group differences and by Dunnett's for intra-group differences with baseline values as the reference time point. Analyses of infarct size, Western blot and mitochondrial calcein fluorescence were performed using one-way ANOVA followed by Student's t-test with Bonferroni's correction for multiple comparisons to avoid type I error. For changes within and between groups a two-tailed p value less than 0.05 was considered significant (SPSS17 for Windows, SPSS Japan, Tokyo, Japan).

Results

Of 189 hearts, 7 were not used secondary to intractable ventricular fibrillation after reperfusion (3 in CTL-45, 2 in CAP-45, 1 in SEVO-45+3MA and 1 in SEVO-45+CAP+3MA) and one due to aortic rupture. Additional hearts were studied until each group had n=8 successful experiments for the infarct size study. The concentration of sevoflurane in the coronary perfusate after 10 min of exposure was 0.25±0.02 mM. Sevoflurane was not detected in the effluent during the baseline, ischemic, and reperfusion periods.

Hemodynamics

Baseline LVDP and CF were similar among the 10 groups. Sevoflurane, chloramphenicol or treatment with 3MA did not significantly affect LVDP or CF before ischemia. After 120 min reperfusion, only SEVO-30 had significantly higher LVDP and lower LVEDP compared to other groups. Treatment with 3MA alone did not affect the recovery of LVDP compared with CTL. There was no significant difference in CF among all the groups throughout experiments. This suggests that changes in CF could not account for improved contractile recovery in SEVO-30 (Table 1).

Myocardial Infarct Size

Myocardial infarct size in SEVO-30 was significantly reduced compared with control hearts (SEVO-30: 24±8% vs. CTL-30: 48±6%, $p<0.05$). However, this effect was lost when the ischemic period was extended to 45 min (SEVO-45: 53±6% vs. CTL-45: 54±11%, p=NS). Chloramphenicol restored protection lost by prolonged ischemia in SEVO-45

(SEVO-45+CAP: 34±8% vs. SEVO-45: 52±10%, $p<0.05$). Restoration of cardioprotection by chloramphenicol was abolished by 3MA (SEVO-45+CAP+3MA: 51±8% vs. CTL-45, p=NS). Treatment with chloramphenicol alone did not limit infarct size (CAP-45: 51±9%) (Figure 2).

Electron Microscopy

Figure 3 shows representative electron microphotographs. Although autophagic vacuoles were rarely seen in cardiomyocytes of CTL (panel A), typical autophagosomes containing intracellular organelles, such as mitochondria and membrane-like structures (white arrows), were apparent after sevoflurane administration (panel B). Additional treatment with chloramphenicol (panel C) further increased the number of autophagosomes. Autophagosomes were diminished by addition of 3MA (panel D).

Western Blot analysis

In SEVO and CAP, LC3-II expression was increased compared with CTL. The combination of sevoflurane and chloramphenicol further increased this expression. These increased expressions of LC3-II were abolished by 3MA (Figure 4). The phosphorylation states of Akt and GSK3β after treatment with sevoflurane, cloramphenicol and 3MA are illustrated by a representative Western blot in Figure 5A. Total Akt and GSK3β were comparable in all samples. The ratio of phospho Akt to total Akt and phospho GSK3β to total GSK3β were significantly increased in SEVO and CAP compared with CTL (Figure 5B). This increase was not caused by unequal loading of the western blot, as shown by α-tubulin detection. The combination of sevoflurane and chloramphenicol enhanced these

increased expressions in SEVO-45+CAP. Administration of 3MA abolished these enhanced expressions in SEVO-45+CAP. There was no significant difference in expression of mitochondrial cyclophilin D between CTL and SEVO-45+CAP (Figure 5C).

Mitochondrial calcein fluorescence

The mitochondrial calcein fluorescence values after treatment with Ca^{2+} were taken as the values from which any reduction in fluorescence was measured. Exposure of mitochondria to 50 μM Ca^{2+} did not induce MPTP opening (Figure 6A). However, exposure of mitochondria to 150 μM Ca^{2+} induced MPTP opening, represented by a reduction in calcein fluorescence of -66.9±13.0% in CTL. This Ca^{2+}-induced reduction in calcein fluorescence was attenuated in the SEVO-45 and CAP-45 groups (-40.0±8.0%, -44.0±1.8% vs. CTL-45, $p<0.05$, respectively; Figure 6B). This effect was enhanced by the SEVO-45+CAP (-15.5±0.3% vs. SEVO-45 and CAP-45, $p<0.05$), which was then abrogated by treatment with 3MA (SEVO-45+CAP +3MA: -74.6±7.8%) (Figure 6B).

Discussion

This study demonstrated that induction of autophagy before ischemia restores the loss of sevoflurane preconditioning cardioprotection with prolonged ischemic insult. This effect is associated with enhanced phosphorylation of GSK3β before prolonged ischemia which results in elevation of the threshold of MPTP opening by Ca^{2+} overload, but not expression of mitochondrial cyclophilin D. There is a consensus that the use of volatile anesthetics during perioperative period could be a promising strategy to reduce ischemia-reperfusion injury for patients at risk of developing cardiac complications. However, the beneficial effects of volatile anesthetics are limited to a certain range of sustained ischemia durations. Thus, strategies that prolong the protective effect of volatile anesthetics during longer ischemic insults should be explored. This study demonstrated for the first time that chloramphenicol treatment could be a novel strategy for this purpose.

Chloramphenicol, used as an antibiotic, is a cytochrome P450 monooxygenase inhibitor which inhibits mitochondrial protein synthesis, causing mitochondrial stress. Chloramphenicol induces autophagy and preconditioning-like cardioprotection, but the mechanisms underlying the cardioprotective effect remains to be established. Thus, we examined whether treatment with chloramphenicol affects expression of mitochondrial cyclophilin D, which is a component of MPTP that controls mitochondrial pore-dependent $Ca2^{+}$ exchange,[18,19] and is a critical mediator of cardioprotection by ischemic preconditioning. There were no significant differences in expression of mitochondrial cyclophilin D between CTL and SEVO-45+CAP. This suggests that cyclophilin D is not involved in cardioprotection by chloramphenicol. Chloramphenicol stimulates the autophagic transcript Atg 12 by inhibiting mitochondrial protein synthesis, which results in

induction of autophagy.[20] This study found that pretreatment with chloramphenicol before ischemia increased expression of LC3-II which leads to formation of autophagosomes. This was confirmed by electron microscopy.

McCormick et al. demonstrated that enhancing autophagy confers protection against ischemia-reperfusion injury in cardiomyocytes.[21] Using *in vivo* swine models, Sala-Mercado et al. showed that induction of autophagy by chloramphenicol before ischemia limited infarct size.[22] However, the precise mechanisms by which pre-ischemic induction of autophagy confers cardioprotection remain to be elucidated. It has been demonstrated that induction of autophagy during ischemia is protective whereas further enhancement of autophagy is detrimental during reperfusion.[23] Thus, it remains controversial whether autophagy is beneficial or harmful in ischemia-reperfusion injury.

MPTP opening is a crucial determinant of ischemia-reperfusion injury.[24] MPTP opening has also been shown to induce autophagy.[25] Elmore et al. demonstrated that autophagic stimulation caused an increase in spontaneously depolarizing mitochondria (presumably transient MPTP opening) in rat hepatocytes.[26] Transient MPTP opening before prolonged ischemia makes the heart more tolerant to ischemia-reperfusion injury.[27,28] We previously demonstrated that heart exposure to sevoflurane increased the threshold of MPTP opening by Ca^{2+} loading.[17] In the present study, exposure of control mitochondria to 150 μM Ca^{2+} induced MPTP opening. This was attenuated in CAP and SEVO, and attenuated to an even greater degree in SEVO+CAP. These data suggests that transient MPTP opening by induction of autophagy with chloramphenicol increases the elevation of the threshold of MPTP opening by sevoflurane alone.

The threshold for MPTP opening is increased by phosphorylation of GSK3β at Ser[9] through phosphatidylinositol 3-kinase (PI3K)/Akt signaling.[16] Whether there is an association between autophagy and GSK3β phosphorylation in reducing ischemia-reperfusion injury is not known. Recently, it was reported that ultraviolet B-induced autophagy activated AMP-activated protein kinase, an important regulator of autophagy through inhibition/phosphorylation of GSK3β.[29] Our data demonstrated that induction of autophagy increased expression of phosphorylated GSK3β at Ser,[9] accompanied by increased Akt phosphorylation. Both sevoflurane and chloramphenicol have been shown to activate PI3K/Akt signaling which is upstream of GSK3β.[17,30] Class III PI3K is involved in the formation of autophagosomes, whereas class I PI3K inhibits the induction of autophagy via phosphorylation of Akt and the mammalian target of rapamycin (mTOR).[31] Furthermore, there is evidence of self-regulation of autophagy by autophagy-induced inhibition of mTOR.[32] Thus, the interaction of Akt with mTOR is multifaceted and bidirectional. Nevertheless, our data suggest that autophagy and PI3K-Akt-mTOR signaling might yield additive benefit against ischemia-reperfusion injury.

In contrast to the in vivo porcine study by Sala-Mercado et al,[22] chloramphenicol alone did not limit infarct size in the present study, despite increased expression of autophagy-related protein LC3-II. However, chloramphenicol-induced autophagy did prolong sevoflurane preconditioning cardioprotection after a longer ischemic insult. Although the reason for the discrepancy remains to be elucidated, differences in ischemia-reperfusion model and species could play a role.

The following study limitations should be acknowledged. First, GSK3β is a substrate of multiple pro-survival protein kinases. We cannot exclude ity that kinases other than

PI3K/Akt signaling contributed to enhanced phosphorylation of GSK3β. Second, the pharmacokinetics of 3MA are not well known, especially at high doses. The dose of 3MA used in this study (10 μM) was low compared to previous studies (5 mM or 5 nM).[21,29] However, this dose has been shown to effectively block LC3-II expression in H9c2 cells as was seen in this study.[33]

In conclusion, induction of autophagy by before ischemia restores the loss of sevoflurane preconditioning cardioprotection with prolonged ischemic insult. This effect is associated with enhanced phosphorylation of GSK3β before prolonged ischemia which is associated with enhanced inhibition of MPTP opening.

3317 words

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Declaration of interest

The authors have no conflicts of interest to report.

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Figure Legends

Figure 1: Schematic illustration of the experimental protocol used in this study. There are ten groups. After 20 min baseline period, isolated perfused hearts were subjected to 30 or 45 min global ischemia and 120min reperfusion. Anesthetic preconditioning was elicited by administration of 10 min of SEVO (2%) with 10 min washout before 30 or 45 min ischemia. CAP and 3-MA was administered starting 20 min before 45 min ischemia and throughout the reperfusion period in hearts from SEVO-treated or non-SEVO-treated animals. Tissue samples were obtained before ischemia. CTL, control; CAP, Chloramphenicol; 3-MA, 3-Methyladenine; SEVO, sevoflurane.

Figure 2: Infarct size as a percentage of LV in ten groups. After 120min reperfusion, infarct size was significantly reduced in SEVO-30 but not in SEVO-45 compared to corresponding CTL. Treatment with CAP in SEVO-45 restored the protection lost by prolonged ischemia in SEVO-45. CAP alone did not limit infarct size after 45 min of ischemia. Restoration of cardioprotection achieved by CAP was abolished by 3MA. 3MA alone did not affect infarct size. Data are presented as mean ± SD; *$p<0.05$ versus CTL-30; †$p<0.05$ versus SEVO-45. CTL, control; CAP, Chloramphenicol; 3-MA, 3-Methyladenine; SEVO, sevoflurane (n=8 for each group).

Figure 3: Electron microphotographs of cardiomyocytes, showing pre-ischemic induction of autophagosomes by reagents. Autophagosomes were rarely seen in the untreated control heart (panel A), were apparently observed (white arrows) after treatment with SEVO-45 (panel B). Autophagosomes appear to be more abundant after additional treatment with CAP

(SEVO-45+CAP) compared with SEVO-45 alone (panel C). Appearance of autophagosomes strikingly diminished after treatment with 3MA (panel D). Nucl, nucleus. Bars, 1 μm.

Figure 4: Representative western blot analysis of LC3-I, II from left ventricular samples obtained before ischemia. The expression of LC3-II was slightly increased in SEVO and CAP compared to CTL. The ratio of LC3-II to LC3-I (LC3-II/LC3-I) was further increased in SEVO-45+CAP compared to SEVO-45 and CAP-45. Administration of 3-MA abolished this increased LC3-II/I in the SEVO-45+CAP group. *$p<0.05$ versus CTL, $^{\dagger}p<0.05$ versus SEVO-45 and CAP-45; CAP, Chloramphenicol; LC3-I, II, microtubule-associated protein light chain I and II; 3-MA, 3-Methyladenine; SEVO, sevoflurane.

Figure 5: (A) Representative western blot of phosphorylated GSK3β from left ventricular samples acquired before ischemia. Expression of phosphorylated GSK3β was significantly increased in the SEVO-45 and CAP-45 group. The combination of SEVO and CAP further increased this expression of phosphorylated GSK3β and Akt in the SEVO-45+CAP group. Administration of 3MA abolished this enhanced expression of phosphorylated GSK3β in the SEVO-45+CAP group.

(B) Densitometric evaluation of four experiments as the *x*-fold increase in average light density *vs.* CTL. The results are presented as the ratio of the phosphorylation state to total protein. The average light intensity was multiplied by 100 to facilitate the presentation of an x-fold increase. Data are presented as mean standard deviation. *$p<0.05$ versus CTL-45, $^{\dagger}p<0.05$ versus SEVO-45, CAP-45 and SEVO-45+CAP+3MA

(C) Cytosolic and mitochondrial expression of cyclophilin D in CTL, CAP-45 and SEVO-45+CAP. There was no significant difference in expression between the three groups.
CTL, control; CAP, Chloramphenicol; CyF D, cyclophilin D; GSK3β, glycogen synthase kinase-3; 3-MA, 3-methyladenine; PHB, prohibitin; SEVO, sevoflurane.

Figure 6: (A) Representative flow cytometric profile of isolated cardiac mitochondria loaded with calcein showing the effects of calcium (50 μM) on mPTP opening as demonstrated by reductions in mitochondrial calcein fluorescence. Exposure of mitochondria to 50 μM Ca^{2+} did not induce MPTP opening in any group. (B) Exposure of mitochondria to 150μM Ca^{2+} induced MPTP opening in CTL. This Ca^{2+}-induced reduction in calcein fluorescence was attenuated in SEVO and CAP. This effect was enhanced by the combination of sevoflurane and chloramphenicol, which was then abrogated by treatment with 3MA. (C) Effect of calcium (150μM) on MPTP opening as demonstrated by reductions in mitochondrial calcein fluorescence. Data are mean ± S.D. Percent change from control in the presence or absence of 3-MA (10 μM). *$p<0.05$ versus CTL-45; $^{\dagger}p<0.05$ versus SEVO-45 and CAP-45 groups. CTL, control; CAP, Chloramphenicol; 3MA, 3-Methyladenine; SEVO, sevoflurane. n=4 for each group

Figure 7: Schematic diagram of potential mechanisms of autophagy-induced cardioprotection conferred by chloramphenicol and sevoflurane. Phosphorylation of GSK3β by PI3-Akt pathway inhibits MPTP opening. Class III PI3K is involved in formation of autophagosomes whereas class I PI3K inhibits the induction of autophagy via phosphorylation of Akt and mTOR. Treatment with chloramphenicol or sevoflurane alone

activates class III PI3K to induce autophagy. Simultaneous treatment with chloramphenicol and sevoflurane enhances this pathway which results in enhanced phosphorylation of GSK3β and elevation of the threshold of MPTP opening.

PI3K = phosphatidylinositol 3-kinase; Akt = protein kinase B; GSK3β = glycogen synthase kinase 3β; mTOR = mammalian target of rapamycin; MPTP = mitochondrial permeability transition pore.

Table 1

TABLE 1. Hemodynamic Variables

	Baseline	Reperfusion (min)		
		30	60	120
LVDP (mmHg)				
CTL-30	109 ± 11	39 ± 8	32 ± 12	26 ± 9
SEVO-30	103 ± 13	61 ± 9*	58 ± 8*	50 ± 8*
CTL-45	108 ± 13	21 ± 6	25 ± 13	24 ± 9
SEVO-45	101 ± 10	23 ± 11	24 ± 10	23 ± 9
CAP+45	109 ± 11	21 ± 7	21 ± 6	21 ± 6
SEVO-45+CAP	101 ± 14	21 ± 5	22 ± 5	21 ± 4
CTL-45+3MA	117 ± 12	18 ± 6	20 ± 7	17 ± 6
SEVO-30+3MA	97 ± 14	44 ± 16†	47 ± 17	44 ± 17
SEVO-45+3MA	95 ± 10	23 ± 15	24 ± 14	20 ± 16
CAP-45+3MA	105 ± 15	25 ± 11	23 ± 10	20 ± 10
CAP-45+SEVO+3MA	97 ± 11	19 ± 9	26 ± 16	26 ± 16
LVEDP (mmHg)				
CTL-30	10 ± 0	47 ± 14	51 ± 18	55 ± 15
SEVO-30	10 ± 0	24 ± 7*	24 ± 5*	26 ± 7*
CTL-45	10 ± 0	67 ± 7	57 ± 13	53 ± 14
SEVO-45	10 ± 0	55 ± 13	50 ± 11	47 ± 12
CAP+45	10 ± 0	53 ± 10	48 ± 10	45 ± 10
SEVO-45+CAP	10 ± 0	53 ± 6	51 ± 10	49 ± 11
CTL-45+3MA	10 ± 0	68 ± 8	66 ± 10	66 ± 11
SEVO-30+3MA	10 ± 0	27 ± 7*	25 ± 9*	25 ± 9*
SEVO-45+3MA	10 ± 0	72 ± 19	62 ± 17	60 ± 19
CAP-45+3MA	10 ± 0	63 ± 12	57 ± 15	52 ± 19
SEVO-45+CAP+3MA	10 ± 0	59 ± 15	51 ± 15	44 ± 16
CF (mL)				
CTL-30	28 ± 6	21 ± 5	21 ± 5	20 ± 8
SEVO-30	31 ± 7	23 ± 4	22 ± 5	22 ± 6
CTL-45	29 ± 7	18 ± 8	18 ± 10	18 ± 9
SEVO-45	28 ± 10	19 ± 11	17 ± 11	17 ± 11
CAP+45	28 ± 3	19 ± 5	18 ± 6	18 ± 7
SEVO-45+CAP	34 ± 4	18 ± 5	19 ± 9	19 ± 9
CTL-45+3MA	37 ± 6	20 ± 8	18 ± 8	18 ± 8
SEVO-30+3MA	29 ± 8	18 ± 7	19 ± 11	20 ± 11
SEVO-45+3MA	27 ± 5	13 ± 5	13 ± 5	11 ± 5
CAP-45+3MA	31 ± 9	20 ± 9	19 ± 9	19 ± 9
SEVO-45+CAP+3MA	31 ± 9	19 ± 7	19 ± 7	18 ± 6

Data are presented as mean ± SD. LVDP=left ventricular developed pressure; LVEDP=left ventricular end-diastolic pressure; CF=coronary flow; CTL=control;CAP=chloramphenicol; 3MA=3-Methyladenine; SEVO=sevoflurane;. *$p<0.05$ vs. CTL-30, †$p<0.05$ vs. SEVO-30 n=8 for each group.

Figure 1

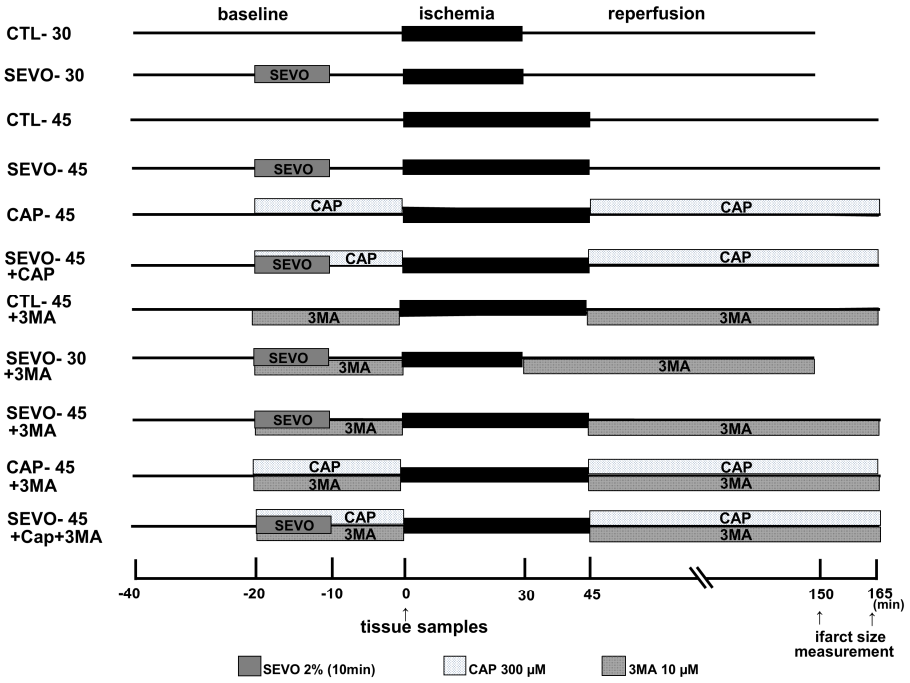

baseline
ischemia
reperfusion
CTL- 30
SEVO- 30
SEVO
CTL- 45
SEVO- 45
SEVO
CAP- 45
CAP
CAP
SEVO- 45 +CAP
SEVO
CAP
CAP
CTL- 45 +3MA
3MA
3MA
SEVO- 30 +3MA
SEVO
3MA
3MA
SEVO- 45 +3MA
SEVO
3MA
3MA
CAP- 45 +3MA
CAP
3MA
CAP
3MA
SEVO- 45 +Cap+3MA
SEVO
CAP
3MA
CAP
3MA
-40
-20
-10
0
30
45
150
165
(min)
tissue samples
ifarct size measurement
SEVO 2% (10min)
CAP 300 μM
3MA 10 μM

Figure 2

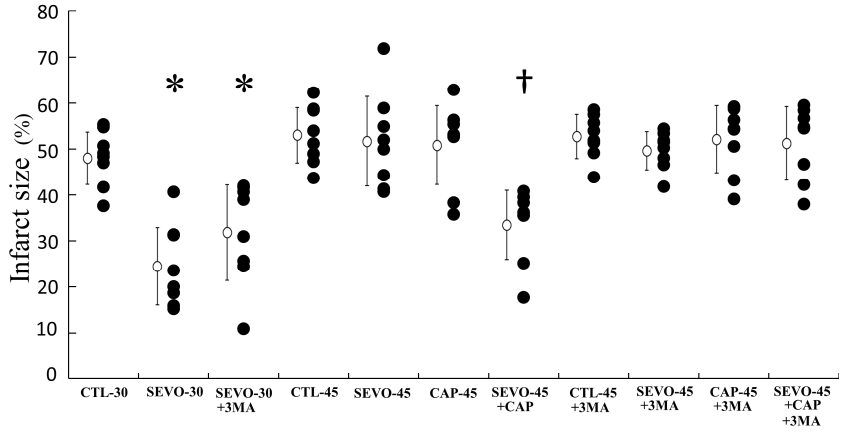

Infarct size (%)
80
70
60
50
40
30
20
10
0
*
*
†
CTL-30
SEVO-30
SEVO-30 +3MA
CTL-45
SEVO-45
CAP-45
SEVO-45 +CAP
CTL-45 +3MA
SEVO-45 +3MA
CAP-45 +3MA
SEVO-45 +CAP +3MA

Figure 3

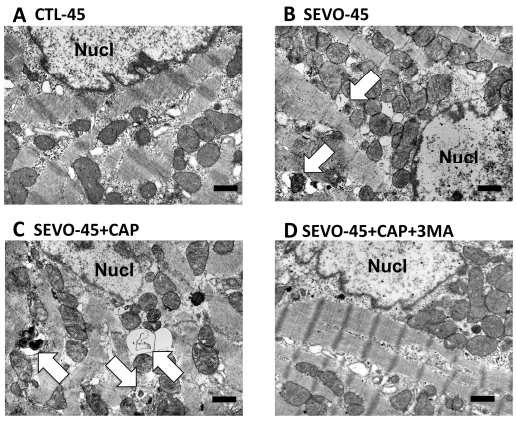

A CTL-45
Nucl
B SEVO-45
Nucl
C SEVO-45+CAP
Nucl
D SEVO-45+CAP+3MA
Nucl

Figure 4

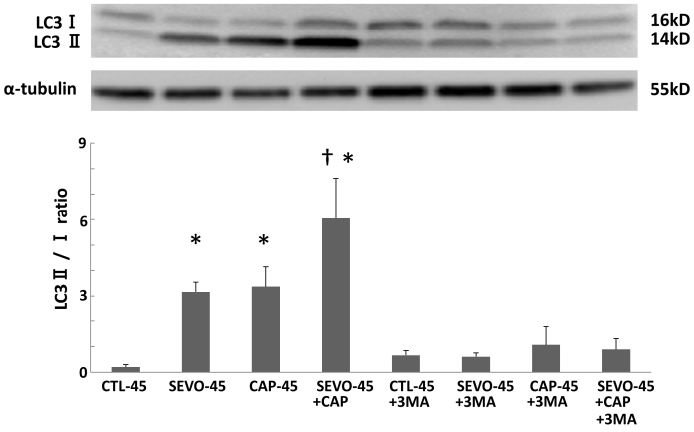

LC3 Ⅰ
LC3 Ⅱ
16kD
14kD
α-tubulin
55kD
LC3 Ⅱ / Ⅰ ratio
9
6
3
0
*
*
† *
CTL-45
SEVO-45
CAP-45
SEVO-45 +CAP
CTL-45 +3MA
SEVO-45 +3MA
CAP-45 +3MA
SEVO-45 +CAP +3MA

Figure 5

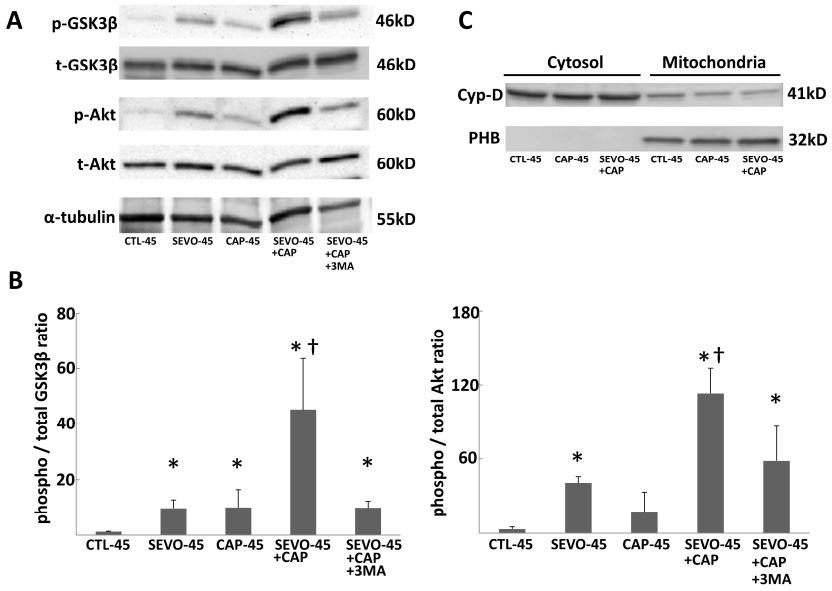

A
p-GSK3β
t-GSK3β
p-Akt
t-Akt
α-tubulin
46kD
46kD
60kD
60kD
55kD
CTL-45
SEVO-45
CAP-45
SEVO-45 +CAP
SEVO-45 +CAP +3MA
C
Cytosol
Mitochondria
Cyp-D
PHB
41kD
32kD
CTL-45
CAP-45
SEVO-45 +CAP
B
phospho / total GSK3β ratio
phospho / total Akt ratio

Figure 6

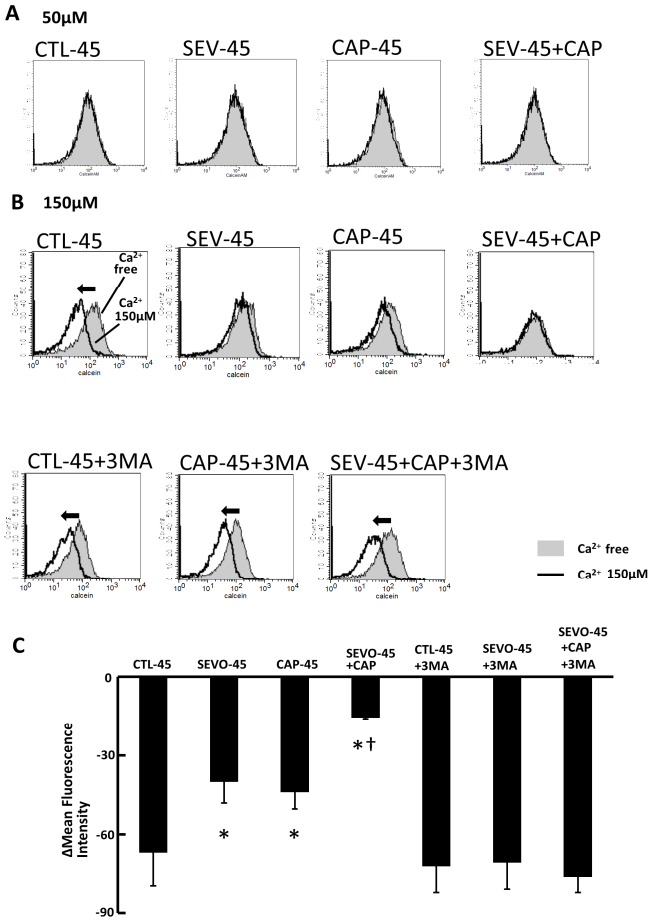
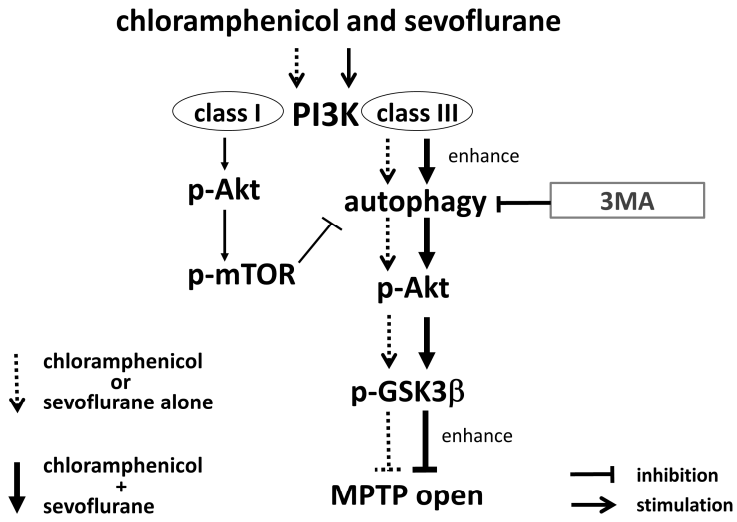

A
50μM
CTL-45
SEV-45
CAP-45
SEV-45+CAP
B
150μM
CTL-45
SEV-45
CAP-45
SEV-45+CAP
Ca2+ free
Ca2+ 150μM
calcein
CTL-45+3MA
CAP-45+3MA
SEV-45+CAP+3MA
Ca2+ free
Ca2+ 150μM
C
CTL-45
SEVO-45
CAP-45
SEVO-45 +CAP
CTL-45 +3MA
SEVO-45 +3MA
SEVO-45 +CAP +3MA
0
-30
-60
-90
ΔMean Fluorescence Intensity
*
*
*†

Figure 7


chloramphenicol and sevoflurane
class I
PI3K
class III
enhance
p-Akt
autophagy
3MA
p-mTOR
p-Akt
chloramphenicol
or
sevoflurane alone
p-GSK3β
enhance
chloramphenicol
+
sevoflurane
MPTP open
inhibition
stimulation